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PHARMACOLOGY AND TOXICOLOGY

The Dependence of the Electrophysiological Effects of Class III Antiarrhythmic Drug Refralon on the Frequency of Myocardium Activation

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We studied the frequency dependence of the effects of the novel Russian class III antiarrhythmic drug refralon on the duration of action potentials (AP) in rabbit ventricular myocardium. The absence of an inverse frequency dependence of AP prolongation was demonstrated: the effects of refralon at stimulation frequency of 1 Hz were stronger than at 0.1 Hz. The patch-clamp experiments with recording of rapid delayed rectifier potassium current I_{Kr} in a heterologous expression system showed that the blocking effect of refralon developed significantly faster at 2 Hz depolarization frequency than at 0.2 Hz. This feature of refralon distinguishes it among the majority of other class III drugs (sotalol, dofetilide, E-4031) and explains the relatively high safety of this drug together with its high efficacy.

Key Words: *action potential; patch clamp; myocardium; delayed rectifier potassium current; antiarrhythmic drug*

Refralon (also known as niferidil, nitro-N-[(1RS)-1-(4-fluorophenyl)-2-(1-ethylpiperidin-4-yl)ethyl]benzamide) is a new class III antiarrhythmic drug produced in Russia and designed for the treatment of atrial tachyarrhythmia. Clinical studies have shown that refralon is highly effective for the relief of both atrial flutter and atrial fibrillation, even in the cases of persisting forms of arrhythmia. In doses of 10-30 μg/kg, the drug restored sinus rhythm in 84.6% patients with atrial fibrillation lasting more than 1.5 months [1] and in 100% patients with atrial flutter [2], whereas dofetilide, a classical class III antiarrhythmic, is effective in only 12.5-30% cases of persisting atrial fibrillation, and amiodarone is effective in 44-48% cases [3-5]. Thus, refralon is superior to all existing class III antiarrhythmic drugs by its effectiveness and has been used in clinical practice for several years. In addition, refralon relatively rare causes polymorphic ventricular tachycardia (1.5% cases) than other class III antiarrhythmic drugs (excluding amiodarone). These promising results of clinical trials have been confirmed by the data of a multicenter study evaluating the efficacy and safety of refralon in real clinical practice in 727 patients [6,7].

The mechanism of the antiarrhythmic effects of refralon remains not studied deeply enough to explain the high efficacy and safety of this drug. Our experiments on mice [8] and guinea pigs [9] showed that refralon caused the most pronounced blocking

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effect on rapid delayed rectifier potassium current I_{Kr} in guinea pig cardiac myocytes (with IC_{50}=1.26 nM) and a slighter effect on acetylcholine-dependent inward rectifier current I_{KACh} (IC_{50}=9.2 μM), while in concentrations >10 μM refralon inhibited almost all major ionic currents in murine and guinea pig cardiomyocytes. Thus, it can be concluded that K_V11.1 (ERG) channels mediating I_{Kr} current are the main target for refralon, though it has not been yet demonstrated directly in experiments with human K_V11.1 channels (HERG). The suppression of I_{Kr} leads to deceleration of repolarization, *i.e.* to lengthening of action potentials (AP), which is the main effect of all class III antiarrhythmic drugs [10].

For the majority of class III antiarrhythmic drugs suppressing I_{Kr} (sotalol, dofetilide, E-4031), an inverse frequency dependence of AP prolongation is observed: these drugs cause much more severe AP prolongation at low frequencies of myocardial activation than at high frequencies [11,12]. This peculiarity of their action is related to high probability of dangerous side effects (ventricular tachycardia). To achieve the desired antiarrhythmic effect (AP lengthening) in fibrillating atria, the drug dose should be increased, which leads to a more pronounced AP lengthening in ventricles and can provoke polymorphic ventricular tachycardia [13].

Our aim was to study the dependence of the effects of refralon on I_{Kr} current in HERG channels expressed in CHO-K1 cells on the frequency of membrane depolarization and to demonstrate the absence of an inverse frequency dependence of refralon effects on AP duration in rabbit ventricular myocardium (papillary muscles). Rabbit myocardium was chosen as the model object due to the prevailing role of delayed rectifier currents I_{Kr} and I_{Ks} in its repolarization, which is also typical of human myocardium.

MATERIALS AND METHODS

Heterologous expression of *KCNH2* gene in immortalized ovarian epithelium cells from Chinese hamster *Cricetulus griseus* (CHO-K1, Chinese Hamster Ovary cells) provided reconstruction of functionally active K_V11.1 channels. The cells were co-transfected with expression vectors *pCI* carrying the gene of interest *KCNH2* and *pMAX* with the *GFP* gene using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific). The fluorescence of the cells expressing GFP served as the marker of successful transfection. The transfected cells were kept under standard culturing conditions in DMEM/F-12 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 2 mM glutamine (Sigma-Aldrich), and 100 μg/ml penicillin-streptomycin (Gibco) at 37°C in an atmosphere containing 21% O_2 and 5% CO_2. The electrophysiological experiments were performed 48 h after transfection.

In transfected CHO-K1 cells, I_{Kr} current was recorded using conventional whole-cell patch clamp method using an Axopatch 200B amplifier (Molecular Devices). A coverslip with cultured CHO-K1 cells was placed into an experimental chamber with a constant flow of physiological saline containing (in mM): 150 NaCl, 3 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES; pH 7.6 adjusted with NaOH at room temperature (23±0.5°C). Only cells with green fluorescence upon exposure to 488 nm wavelength light were chosen for the experiments. Patch pipettes with tip resistance 2-3 MΩ were pulled from borosilicate glass capillaries (Sutter) and filled with a solution containing (in mM): 140 KCl, 1 $MgCl_2 \times 6H_2O$, 5 EGTA, 4 Mg_2ATP, and 10 HEPES; pH 7.2 adjusted with KOH [14]. Before current recording, the pipette capacitance, the capacitance of the studied cell, and the access resistance were compensated. During data analysis, the current amplitude was normalized by the cell capacitance and expressed as pA/pF. I_{Kr} was recorded using a standard two-step protocol with depolarization from the holding potential of -80 mV (Fig. 1). The second step, repolarization to -40 mV, was necessary for registration of I_{Kr} tail current, the amplitude of which corresponds to the current magnitude in the absence of C-type inactivation. The effects of refralon were assessed by the peak values of the tail current.

For experiments on rabbit myocardial cell preparations, 15 adult (2.5-3-months-old) male Soviet Chinchilla rabbits weighing 2.8-3.1 kg were used. The animals were anesthetized with urethane (1.5 g/kg, intravenously), the heart was excised and rinsed with Tyrode solution (in mM: 133.47 NaCl, 4.69 KCl, 1.35 $NaH_2PO_4 \times 2H_2O$, 16.31 $NaHCO_3$, 1.18 $MgSO_4 \times 7H_2O$, 2 $CaCl_2 \times 2H_2O$, and 7.77 glucose) aerated with carbogen (95% O_2, 5% CO_2). The right ventricle was opened and the papillary muscle preparation was isolated and mounted into a 3-ml experimental perfusion chamber (flow rate 10 ml/min, 38°C). The preparation was stimulated with electrical field through silver teflon-coated electrodes (pulse duration 2 msec, amplitude 2-fold surpassed the threshold value for each preparation).

AP were recorded by a standard method of intracellular registration of bioelectrical activity using sharp glass microelectrodes with tip resistance 25-40 MΩ. The signal from Neuroprobe 1600 amplifier (A-M Systems) was recorded using Powergraph 3.3 software (DiSoft). During data analysis, AP duration at the level of 90% repolarization was evaluated (APD_{90}).

The statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software). The data are presented as *M*±*SEM*. The statistical

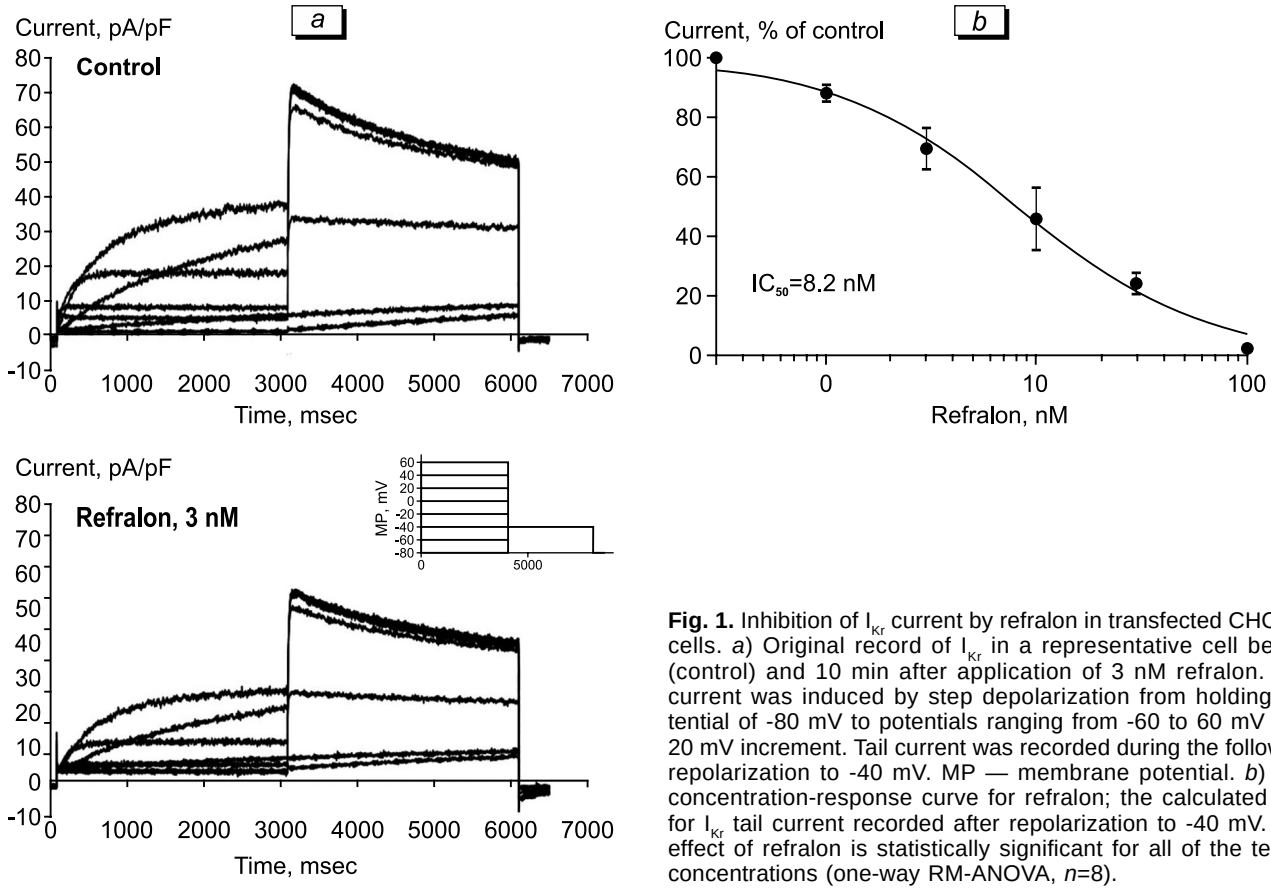

Fig. 1. Inhibition of I_{Kr} current by refralon in transfected CHO-K1 cells. *a*) Original record of I_{Kr} in a representative cell before (control) and 10 min after application of 3 nM refralon. The current was induced by step depolarization from holding potential of -80 mV to potentials ranging from -60 to 60 mV with 20 mV increment. Tail current was recorded during the following repolarization to -40 mV. MP — membrane potential. *b*) The concentration-response curve for refralon; the calculated IC_{50} for I_{Kr} tail current recorded after repolarization to -40 mV. The effect of refralon is statistically significant for all of the tested concentrations (one-way RM-ANOVA, n=8).

significance of the effects of refralon on AP at different stimulation frequencies was detected using two-way ANOVA with Sidak's post-hoc test. The relative APD_{90} increase in the two groups of myocardial preparations (n=5 in each group) was compared using the Student's t test. The differences were significant at $p<0.05$.

RESULTS

I_{Kr} current mediated by HERG channels expressed in a heterologous expression system (CHO-K1 cells) appeared to be highly sensitive to refralon. Even in a concentration of 1 nM, refralon caused a statistically significant decrease in tail current amplitude ($p<0.05$), while in a concentration of 3 nM it reduced the current by more than 25% (Fig. 1, *a*, *b*). As the inhibiting effect after application of refralon developed slowly, each concentration of the drug was applied for at least 10 min to achieve the full effect. The calculated IC_{50} for I_{Kr} inhibition by refralon was 8.2 nM. Thus, the sensitivity of I_{Kr} mediated by human channels expressed in a heterologous system to refralon was comparable to that for the same current in guinea pig atrial myocytes (IC_{50}=1.26 nM). Based on the obtained concentration–response curve, 30 nM refralon was chosen for further experiments on CHO-K1 cells, because this concentration provided strong, but incomplete inhibition of I_{Kr}.

To analyze the influence of HERG channels stimulation frequency on the magnitude of the inhibiting effect of refralon we performed 2 series of experiments with cell depolarization at a frequency of 2 and 0.2 Hz, respectively. The cells were stimulated with 250 msec depolarizing steps (0 mV), which approximately corresponds to the duration of ventricular AP. The duration of the series of steps was 2 min; prior to and at the end of refralon application, a standard 2-sec depolarization was applied to evaluate the effectiveness of refralon application. The inhibition of I_{Kr} by refralon was significantly more pronounced in cells depolarized at a frequency of 2 Hz ($p<0.05$) (Fig. 2). One can conclude that effect of refralon develops faster at high stimulation frequencies. To evaluate the influence of depolarization frequency on the effect of refralon on the rate of repolarization in the myocardium, we performed additional experiments on tissue preparations of rabbit myocardium.

The rate of repolarization in the ventricular myocardium of rabbit papillary muscles strongly depends on the activation rate. Increasing the frequency from 0.1 to 1 Hz leads to a pronounced AP lengthening, *i.e.* a deceleration of repolarization (Fig. 3) [15]. Therefore,

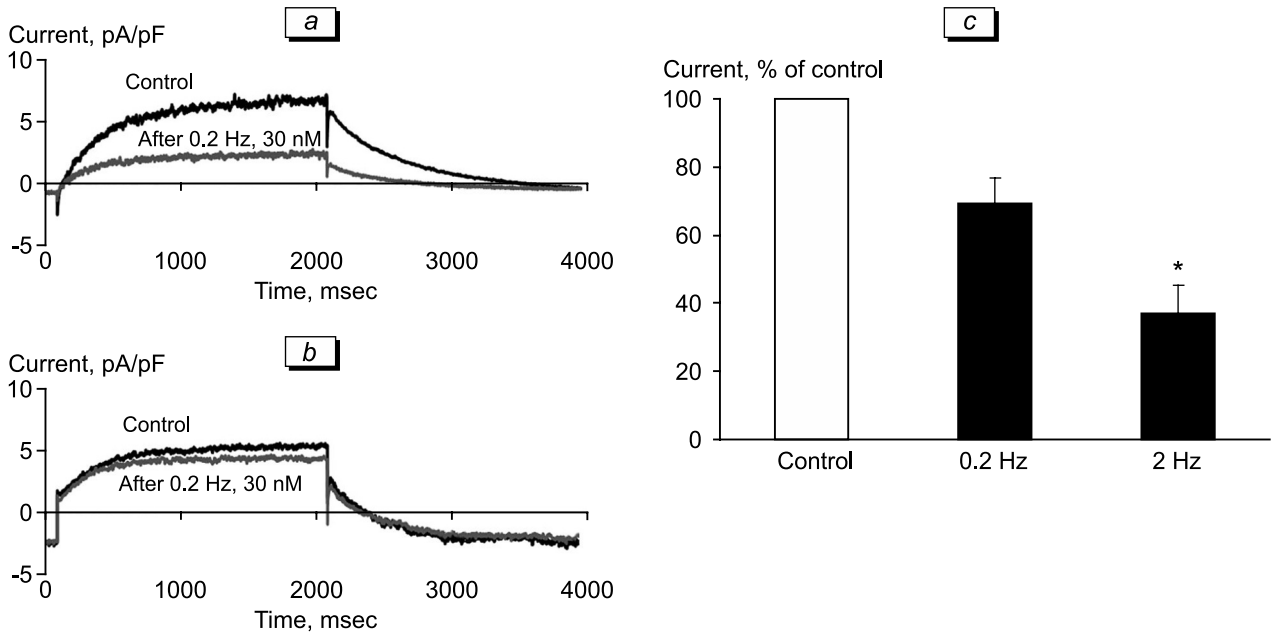

Fig. 2. Effect of cell depolarization frequency on the efficacy of inhibition of I_{Kr} by refralon. *a*, *b*) Representative records of I_{Kr} induced by a 2-sec depolarizing step from holding potential of -80 to 0 mV before (control) and 2 min after the application of 30 nM refralon with repetitive background depolarizing steps to 0 mV at 2 Hz (*a*) and 0.2 Hz (*b*) frequency. The duration of the depolarizing steps during the refralon application was 250 msec. *c*) Comparison of the inhibitory effect of refralon after depolarization at 2 Hz (n=8) and 0.2 Hz (n=8) frequency. *p<0.05 in comparison with 0.2 Hz (Student's t test).

in each experiment we measured APD_{90} before and after application of refralon at 6 different frequencies of stimulation ranging from 3 to 0.1 Hz (Fig. 3, *c*-*e*). During refralon application, 3 groups of preparations were stimulated at the frequencies of 0.1 Hz (n=6), 1 Hz (n=6) and 3 Hz (n=5), respectively, for 20 min. A higher concentration of refralon (300 nM) was chosen for the experiments on tissue preparations, because additional diffusion barriers in the myocardium hamper drug availability for cells and limits its effect [9].

In most preparations refralon caused a moderate AP lengthening (Fig. 3, *a*) that did not exceed 70-80% of APD_{90} under control conditions (Fig. 3, *c*-*e*). In two preparations (from groups stimulated at 0.1 and 1 Hz), a multiple increase in APD_{90} occurred during AP registrations at 6 different frequencies after refralon application. In one of these preparations, it was accompanied by arrhythmogenic activity including early afterdepolarizations. These preparations were excluded from the samples due to their atypical reaction to refralon and large APD_{90} at basal conditions (>270 msec).

At all three tested stimulation frequencies, refralon caused a significant increase in APD_{90} (Fig. 3, *c*-*e*). The relative AP elongation was evaluated at similar frequency (1 Hz) for the three groups (Fig. 3, *b*). The effect of refralon observed at 1 Hz stimulation frequency was significantly (p<0.05) stronger than that in the group of preparations stimulated at 0.1 Hz frequency. No significant differences were detected for the group of preparations stimulated at 3 Hz. Thus, refralon at least does not demonstrate inverse frequency dependence of the developed effect, although the obtained data are insufficient to claim the presence of a direct frequency dependence.

The obtained results allow us to presume that the development of refralon effects requires prolonged rhythmical stimulation of the myocardium; at the cellular level, repetitive opening of $K_V11.1$ channels is required. Further experiments should clarify whether the drug is capable of binding to resting and inactivated channels, or it binds only to channels in the opened state. We have now proved that the effects of refralon on I_{Kr} current and AP configuration considerably differ from that of class III antyarrhythmic drugs E-4031, dofetilide, and sotalol that are characterized by an inverse frequency dependence of the AP lengthening caused by these drugs. It is well known that binding of these drugs to $K_V11.1$ channels requires channel opening; however, the inhibiting effect reaches its maximum only after a few depolarizations of the cell [11,15]. On the contrary, I_{Kr} inhibition by refralon takes several minutes to develop even under condition of patch clamp experiment, and high frequency depolarization of the cells is required to speed up the blockade of I_{Kr}. Thus, low frequency stimulation of the myocardium is enough to cause the inhibition of I_{Kr} by E-4031 [15], while the effect of refralon can

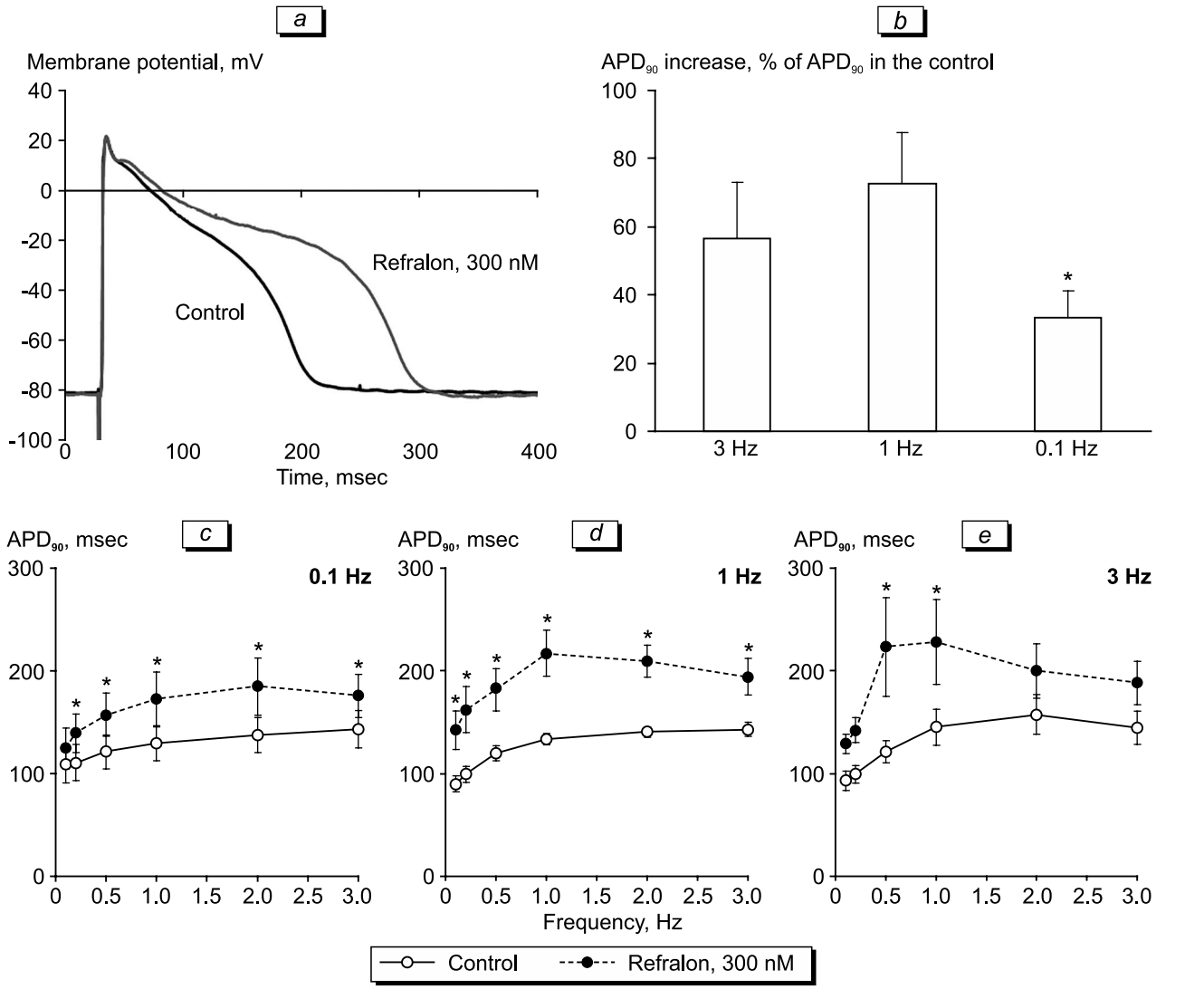

Fig. 3. Effect of the frequency of stimulation of the papillary muscles from rabbit right ventricle on deceleration of repolarization caused by 300 nM refralon. *a*) Original record AP in a representative preparation of rabbit right ventricle papillary muscle before (control) and after application of 300 nM refralon over 20 min with background stimulation of the preparation at 1 Hz. *b*) Comparison of APD_{90} repolarization caused by 300 nM refralon under condition of stimulation of the preparations at 3, 1, and 0.1 Hz (n=5 for each group). For adequate comparison, AP were recorded under 1 Hz stimulation in all experimental series. *p<0.05 in comparison with 1 Hz (Student's t test). *c*-*e*) The frequency dependence of APD_{90} before (control) and after application of 300 nM refralon under conditions of stimulation of the preparations at 0.1 Hz (*c*; n=5), 1 Hz (*d*; n=4), and 3 Hz (*e*; n=5) frequency. *p<0.05 (two-way ANOVA with Sidak's post-hoc test).

be achieved faster at high activation frequencies. As a consequence, AP prolongation caused by E-4031 would be achieved faster at low frequencies of myocardium excitation. In case of atrial fibrillation, a higher dose of the drug would be required to achieve the necessary therapeutical effect – and, thus, it will increase the risk of excessive AP lengthening in ventricular cardiomyocytes and occurrence of early afterdepolarizations. At the same time, refralon will cause at least similar deceleration of repolarization both in ventricles and in fibrillating atria, producing a therapeutic effect at relatively safe concentrations. Further studies will allow to test this hypothesis and to clarify the molecular mechanisms mediating the revealed dependence of the effects of refralon on the frequency of myocardial activation.

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